ENCL. 1

Eur J Clin Pharmacol (2005) 60: 859–864
DOI 10.1007/s00228-004-0850-0

PHARMACOKINETICS AND DISPOSITION

Davide Soldini · Hugo Zwahlen · Luca Gabutti
Antonio Marzo · Claudio Marone

Pharmacokinetics of *N*-acetylcysteine following repeated intravenous infusion in haemodialysed patients

Received: 13 May 2004 / Accepted: 23 September 2004 / Published online: 19 January 2005

Abstract *Objective*: *N*-acetylcysteine (NAC) is a mucolytic agent with anti-oxidant properties. It might have potential positive effects in renal patients and, therefore, its pharmacokinetics and safety in haemodialysis was investigated.
Methods: Twelve dialysis patients received 2 g NAC (10 ml NAC 20% solution i.v.) mixed with 500 ml saline during the first 3 h of the session for six dialysis sessions. A bolus of heparin was injected intravenously as LWH-heparin. In six patients, one session was repeated with NAC mixed with heparin and infused through the heparin pump.
Results: Baseline NAC was on average 454 ng ml^{-1}; its concentration increased to 9,253 ng ml^{-1} at the second infusion and attained a steady state between 14,000 ng ml^{-1} and 17,000 ng ml^{-1} at the fourth dose. We observed a C_{max} of 53,458 ng ml^{-1} with a t_{max} of 3.0 h. Plasma clearance was 1.25 l h^{-1} and dialytic clearance 5.52 l h^{-1}. No side effects were observed.
Conclusion: In the case of repeated doses, the NAC pre-dose concentration after repeated infusion of 2 g of the drug during the first 3 h of a dialysis session reached the steady state at the fourth infusion, without further accumulation. The dialytic clearance is effective, the total body clearance being reduced to 1.25 l h^{-1}. In dialysis patients, 2 g NAC given intravenously over 3 h is a safe dosage, with no short-term side effects.

The study was conducted in accordance with the guidelines for Good Clinical Practice and all current Swiss laws concerning clinical research. The protocol was approved by the Ethics Committee of the Canton Ticino, Switzerland.

D. Soldini · H. Zwahlen · C. Marone (✉)
Division of Nephrology,
Ospedale Regionale di Bellinzona e Valli,
6500 Bellinzona (TI), Switzerland
E-mail: claudio.marone@eoc.ch
Tel.: +41-91-8119109
Fax: +41-91-8118799

L. Gabutti
Division of Nephrology,
Ospedale Regionale di Locarno,
6600 Locarno (TI), Switzerland

A. Marzo
Clinical Pharmacology Unit, IPAS SA,
6853 Ligornetto (TI), Switzerland

Introduction

Patients with end-stage renal insufficiency, on regular dialysis treatment, are prone to suffer from cardiovascular diseases and show a high mortality due to stroke and ischaemic heart disease [1]. Dialysis patients present various risk factors for atherosclerosis, such as hyperlipidemia, hyperhomocysteinaemia, abnormalities of glucose and calcium metabolism, hypertension, hyperuricaemia and mytogenic factors that can stimulate smooth muscle proliferation [1]. Polymorphonuclear cell-mediated oxidant injury is also an important mechanism of endothelial dysfunction in dialysed patients, which leads to adverse cardiovascular events [2–8].

Elevated oxidative stress has been related to increased cardiovascular risk in dialysis patients. In fact, oxidative stress in this population is higher in patients with cardiovascular diseases than in those without [9, 10].

Hypoalbuminaemia is the most powerful predictor of mortality in end-stage renal diseases, and it is mostly caused by decreased albumin synthesis [11]. As albumin concentration and synthesis are primarily determined by non-nutritional factors, a positive effect of *N*-acetylcysteine (NAC) on the albumin level in cachectic patients seems possible, probably through a change in the redox state [11].

N-acetylcysteine is a mucolytic agent, which plays a primary role in the maintenance of adequate GSH (reduced form of glutathione) levels, contributing to the cellular protection against harmful agents [12, 13]. Being an anti-oxidant agent, NAC might be potentially useful in dialysis patients. A previous study by Tepel et al. [14] in chronic renal failure patients, who received 600 mg

BID of NAC orally for 24 months, reported of a decrease in the achievement of primary end-points (cardiac events, ischaemic stroke, peripheral vascular disease) in comparison with patients on placebo, with no difference on cardiovascular or total mortality. As a pilot study, we wanted to investigate the pharmacokinetics and safety of a higher dose of NAC given in haemodialysed patients as an intravenous (i.v.) infusion with a dosing schedule of 2 g over 3 h during dialysis sessions, which will be considered for future clinical trials.

Materials and methods

Subjects

During the first phase, we intended to recruit 12 end-stage renal disease patients, who underwent chronic maintenance haemodialysis three times a week, for at least 6 months. They should not have been suffering from an uncontrolled arterial hypertension, not have been prone to hypotensive episodes nor have presented with clinically significant diseases as determined by history, physical examination and laboratory tests. Patients, who participated in other trials within the previous 3 months, were alcohol-, drug- or nicotine-addicted or were overweight (> 15% of ideal body weight) were excluded. During the second phase, we intended to repeat the pharmacokinetic study day in a subgroup of patients, with a modified NAC administration modality.

Dosing and sample collection

Dialysis was performed three times per week with a session duration of 4 h. All patients were dialysed with a Fresenius 4008H haemodialysis machine using a Fresenius F8 polysulfone 1.8-m^2 dialyser. Blood flow (250 ml min^{-1}) and dialysate flow (500 ml min^{-1}) were kept constant.

This study was conducted in two phases. The first phase considered 12 patients and lasted for six consecutive dialysis sessions with NAC infusions, with all predose samplings and a curve lasting 48 h after the last infusion (dialysis 6). The second phase was carried out on 6 of the 12 patients from phase one and concerned only one dialysis session with NAC infusion.

This study was a clinical, open, one-way trial, with repeated individual doses every 2–3 days. After a run-in period of 1 week, a dialysis was performed without NAC infusion (dialysis 0), and blood was sampled at 0 h. Then during the next dialysis, 2 g NAC (10 ml NAC 20% solution i.v.) mixed with 500 ml saline solution was infused into the venous line (after the dialyser) during the first 3 h of the session. A bolus of heparin, needed for anti-coagulation during haemodialysis, was given i.v. as LMW-heparin. The same infusion has been repeated during the successive five haemodialyses. During the infusion, blood pressure and heart rate were continuously monitored. Blood samples were collected during the last dialysis with NAC administration for the pharmacokinetic analysis of NAC (dialysis 6: at times 0, 3, 3.5, 4, 6, 9, 12, 24 and 48 h). Furthermore, the other days when NAC was administered (dialysis 1, 2, 3, 4, 5) blood samples were taken at time 0 (beginning of the infusion). During the last dialysis, blood samples for drug assay and haematocrit were taken at times 3.0, 3.5 and 4.0 h simultaneously from the arterial and the venous lines. A follow-up sample was taken 26 days after the beginning of the study.

After having excluded interactions between NAC and heparin in vitro, we modified the administration modality of NAC. The experiment was repeated in six patients as in dialysis 6 with 2 g NAC, and the required heparin mixed and dissolved into 30 ml saline and infused over a 3-h period through the heparin pump, which was already integrated into the dialysis machine prior the dialyser.

Plasma concentrations were evaluated for total glutathione and total cysteine at dialysis 0, 1, 2, 6 predose and at follow-up, and plasma concentration of blood urea nitrogen (BUN) at dialysis 0 pre-dose, at dialysis 1 pre-dose and 4 h, dialysis 2 pre-dose, dialysis 6 pre-dose, at 3 h and 4 h and at follow-up.

This protocol was approved by the ethics committee of the Canton Ticino, Switzerland.

Bioassays

N-acetylcysteine was assayed in plasma by means of a high-performance liquid chromatography (HPLC) method with ultraviolet (UV) detection. The method involved the derivatisation of NAC with *N*-(4-anilinophenyl) maleimide after reduction of the oxidized thiolic groups of NAC with dithiothreitol. *N*-propionyl-L-cysteine was used as internal standard. The chromatography was performed on a ODS reverse-phase column, and the effluent was monitored using UV detection at 290 nm. The method was validated over the concentration range 50–1,000 ng ml^{-1} using 0.1 ml plasma. Both intra- and inter-assay precision and accuracy for quality control (QC) values at three concentration levels (900, 500, 150 ng ml^{-1}) proved to be within 5.0%. Specificity was good, since no interfering peaks from the matrix were detected. The limit of quantification was 50 ng ml^{-1} with coefficient of variation (CV) values of 1.4% and 8.9% in the intra- and inter-assay tests, respectively.

Total glutathione and total cysteine were assayed in plasma using a fully validated HPLC method with fluorimetric detection. BUN was assayed in the hospital laboratory according to the operating routinary method.

Pharmacokinetic analysis

For each subject and each treatment, the following pharmacokinetic parameters were considered:

C_{max} (ng ml^{-1})	Peak concentration (maximum plasma concentration)
t_{max} (h)	Time to peak plasma concentration
AUC_{48} (ng ml^{-1} h)	Area under the curve
Cl_p (l h^{-1})	Total plasma clearance

All pharmacokinetic parameters were directly extrapolated (C_{max}, t_{max}) or calculated (AUC_{48}, Cl_p) from the individual concentration–time data using the program WinNonlin provided from Pharsight. The area under the concentration–time curve from the time of dosing to the last quantifiable concentration was calculated by mean of the linear trapezoidal rule.

Dialytic clearance

The dialysis clearance (Cl_D) of NAC was calculated using the arterio-venous difference method, according to the following formula:

$$Cl_D = Q_b(1 - A_{Hct})\frac{\left(A_p - V_p\left[1 - \frac{V_{Hct} - A_{Hct}}{V_{Hct}}\right]\right)}{A_p}$$

where Q_b equals blood flow, A_{Hct} haematocrit of arterial-line blood, A_p arterial-line plasma concentration (before dialyser), V_p venous-line plasma concentration (after dialyser) and V_{Hct} haematocrit of venous-line blood.

Statistics

All pharmacokinetic parameters were summarized by treatment and were subjected to descriptive statistics, in order to have mean and standard deviation. Analysis of variance was carried out on ln-transformed variables, using WinNonlin from Pharsight.

Results

In three chronic in-centre haemodialysis programs, 13 patients (10 males and 3 females) gave informed consent to participation to the study. The first patient had to be replaced because after the first session he received a kidney transplant. The 12 patients who completed the study had a mean (±SD) age of 57±9 years and weight of 78±8 kg. The patients' characteristics are listed in Table 1. Of these patients, 6 gave their consent also for the second phase of the study.

Pre-dose plasma concentrations of NAC are presented in Fig. 1. Baseline NAC concentration on study day 0 was on average 454±193ng ml^{-1} (mean±SD). The pre-dose NAC concentration before the first administration was 1,937 (±2,212) ng ml^{-1}. This was caused by three patients who showed values markedly higher than the other patients. Pre-dose NAC concentration increased to 9,253±4,162 ng ml^{-1} at the second infusion (dialysis 2) and ranged on average from 14,000 to 17,000 ng ml^{-1} in further pre-dose samples. The first

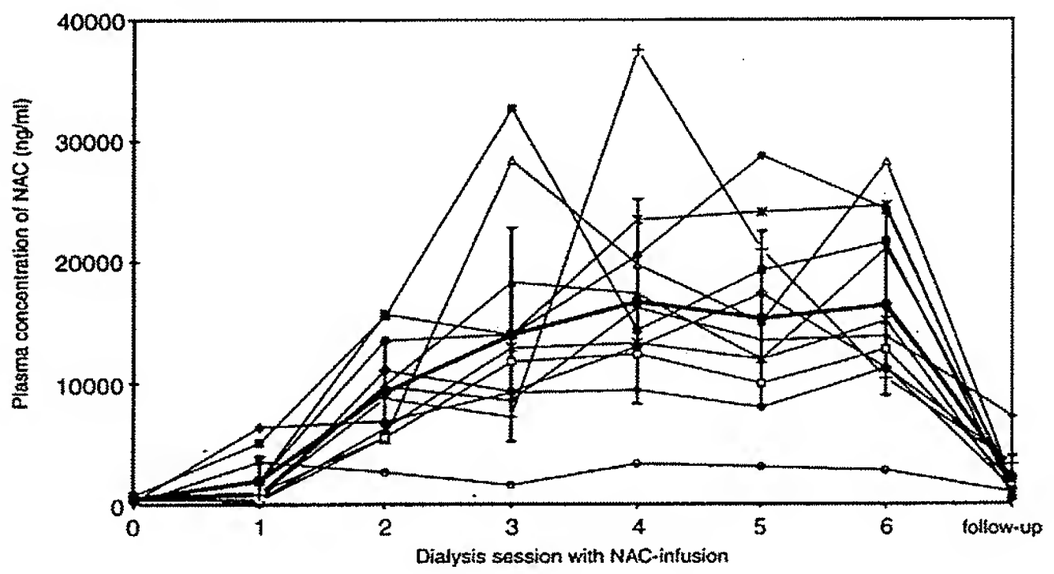

Fig. 1 Baseline (dialysis 0), pre-dose and follow-up concentrations of *N*-acetylcysteine after a 3-h infusion of 2.0 g of the drug in the 12 subjects. Mean (*thick line*) and standard deviation (*bars*) are also given.

Table 1 Demographic data and main concurrent illnesses of the enrolled patients

Subject	Sex	Age (years)	Height (cm)	Weight (kg)	Main concurrent illnesses
1	Male	76	173	67.0	Hypertension, osteopathy, anaemia, peripheral arteriopathy
2[a]	Male	51	172	81.4	Epilepsia, hypertension, anaemia, hypercolesterolaemia
3[a]	Female	61	167	82.0	Anaemia, hypertension
4[a]	Male	67	173	74.4	Anaemia, hypertension
5[a]	Male	54	162	83.0	Diabetes mellitus, anaemia, hypertension
6	Female	51	174	88.0	Hypertension, osteopathy, anaemia
7	Male	54	176	87.0	Hypertension, anaemia, diabetes insulin-dependent
8	Male	65	167	69.0	Megaloblastic anaemia
9	Female	51	170	66.0	Hypertension, anaemia, ischaemic cardiomyopathy
10[a]	Male	46	178	78.0	Hypertension
11[a]	Male	48	179	83.0	Anaemia, hypertension, osteopathy, peripheral arteriopathy
12	Male	55	162	81.0	Hypertensive cardiopathy, anaemia, osteopathy, gastritis, chronic obstructive bronchopathy

[a]These patients participated also in the second phase of the trial

pre-dose concentration (day 0) was statistically different ($P < 0.001$) than all other pre-dose values. The steady state of pre-dose concentration was reached at the fourth dose of NAC. The blood concentration of NAC continuously decreased in the follow-up period, but it was still four times higher than baseline (2,129 versus 454 ng ml^{-1}) 14 days after the last dose. Concerning pharmacokinetics, we found a C_{max} of 53,458 ± 12,998 ng ml^{-1} with a t_{max} of 3.0 ± 0.0 h and an AUC_{48} of 1,094,336 ± 326,797 ng ml^{-1} h (Table 2). Plasma clearance of NAC was 1.25 l h^{-1}, which corresponds to 0.019 l h^{-1} kg^{-1}. Dialytic clearance of NAC measured at times 3.0, 3.5 and 4.0 h after the start of the infusion at the dialysis 6 was on average 5.52 l h^{-1}.

In Fig. 2, we compare the pharmacokinetic results of the first and second phases of the study for the six patients who repeated the experience. We observe smaller C_{max} and AUC_{48} (about 50%) values during the second phase, but a higher Cl_p and comparable Cl_D.

The dialytic clearance of BUN, calculated from arterial/venous plasma concentration at 4 h at dialysis 6, was on average 8.9 l h^{-1} and 8.7 l h^{-1} during the first and second study phases, respectively. Plasma concentration of cysteine ranged on average 36–41 μg ml^{-1} in baseline, pre-dose and follow-up samples. Plasma concentration of glutathione ranged on average 1.45–1.56 μg ml^{-1} in baseline and in pre-dose samples and increased to 1.94 μg ml^{-1} and to 1.80 μg ml^{-1} during the follow-up period after the first and second study phases. Safety was good in all the cases.

Table 2 Pharmacokinetic parameters of *N*-acetylcysteine (ng ml^{-1}) for the first study phase, after the sixth 3-h infusion of 2.0 g of the drug. C_{max} peak plasma concentration, *AUC* area under the plasma concentration–time curve, Cl_p plasma clearance, Cl_D dialytic clearance

Patient	C_{max} (ng ml^{-1})	t_{max} (h)	AUC_{48} (ng ml^{-1} h)	Cl_p (l h^{-1})	Cl_D (l h^{-1})
1	45,580	3.0	844,892	1.72	4.56
2	77,414	3.0	1,423,417	0.78	5.47
3	56,630	3.0	1,327,713	1.03	5.63
4	70,597	3.0	862,747	1.16	4.58
5	45,693	3.0	1,129,531	1.01	6.65
6	56,982	3.0	1,541,558	0.51	5.53
7	62,623	3.0	1,439,179	0.62	5.76
8	29,732	3.0	440,794	4.02	3.56
9	41,623	3.0	954,057	1.08	7.50
10	50,949	3.0	1,190,579	0.75	5.25
11	56,701	3.0	1,209,738	0.75	6.39
12	46,972	3.0	767,831	1.58	5.39
Mean	53,458	3.0	1,094,336	1.25	5.52
SD	12,998	0.0	326,797	0.94	1.03

Discussion

In the case of the repeated dose regimen, the pre-dose concentrations are an expression of accumulation and/or steady-state achievement. Our study demonstrates that the pre-dose concentration after a repeated infusion of 2 g NAC during the first 3 h of a dialysis session at first increases and then reaches the steady state at the fourth infusion, without further accumulation (mean concentration between 14,000 ng ml^{-1} and 17,000 ng ml^{-1}). The dialytic clearance is effective for both NAC and BUN, being most effective for the latter. The total body clearance of NAC was instead reduced to 1.25 l h^{-1} corresponding—for a mean body weight of 65 kg—to 0.019 l h^{-1} kg^{-1}. In non-dialysed subjects, much higher total body clearances were measured (Holdiness [15] reported 0.11 l h^{-1} kg^{-1} and Jones et al. [16] 0.10 l h^{-1} kg^{-1}). Dialytic clearance during the second phase was similar (5.66 l h^{-1} versus 5.27 l h^{-1}). The difference between the dialytic and total clearance arises from the different measurement intervals (1 h for dialytic clearance and 48 h for total clearance with the dialysis operating only for 4 h). The concentration of NAC in fact continuously decreases during the follow-up period, but it was still four times higher than baseline 14 days after the last dose. No side effects were observed, such that NAC 2 g infused i.v. for 3 h during dialysis can be considered well tolerated and safe within the limits of the small number of patients in the study. During the first phase, NAC was infused in a venous line separated from heparin, which was given i.v. as a bolus as LMW-heparin; during the second phase, NAC was mixed with heparin and then given through the heparin pump. The comparable results concerning pre-dose concentrations during both phases show that there are

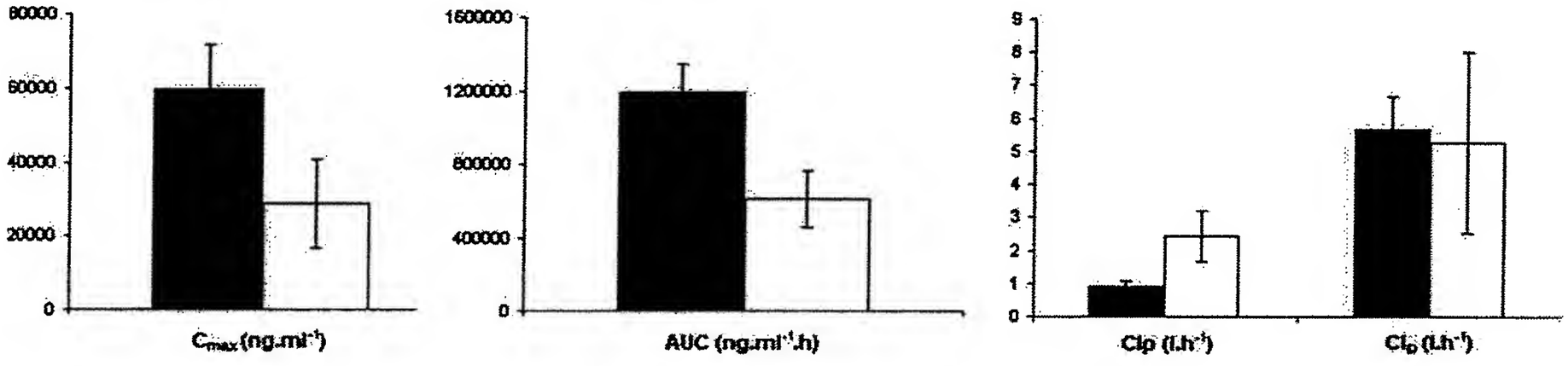

Fig. 2 Peak plasma concentration (C_{max}), area under the plasma concentration–time curve (AUC), plasma clearance (Cl_p) and dialytic clearance (Cl_D) in the six patients who were studied during both the first phase (heparin in bolus i.v. as LMW-heparin prior to and NAC infused after the dialyser—*grey bars*) and the second phase of the study (heparin i.v. mixed with NAC given prior to the dialyser—*white bars*)

no important interactions between heparin and NAC and that the administration via the heparin pump is a suitable option. The comparison of dialytic clearance with plasma clearance indicates that NAC is effectively cleared from the body by the dialysis process.

In the rare cases of patients with paracetamol poisoning accompanied by renal insufficiency, the current therapeutic strategy with NAC is inappropriate and needs to be modified. While the first dose can be given unchanged as loading dose (150 mg kg^{-1} in 15 min), the second (50 mg kg^{-1} in 4 h) and the third doses (100 mg kg^{-1} in 16 h) should be reduced by one-third since approximatively 70% of the total body clearance of NAC is non-renal [15]. In the case of haemodialysis, a supplement dose should be added at the end of the dialysis session. Considering a dialytic clearance of 5.52 l h^{-1} and a volume of distribution of 0.40 l kg^{-1} [15], we obtained an elimination constant (k) of 0.21 h^{-1}. For a session of 4 h, the removed dose (also corresponding to the supplement dose) results

$$1 - e^{-kt} = 1 - e^{-(0.21)(4)} = 57\%$$

of the given dose; in other words, about half of the amount of NAC given during dialysis has to be replaced.

Regarding pharmacokinetics in both study phases, a statistical comparison is not possible since the modality of NAC administration was different; during the first, NAC was infused via the venous line, after the filter; whereas, during the second phase, NAC was given with heparin prior to the dialyser and, therefore, the drug underwent a "first-pass" effect. The distinct administration modality explains most of the differences in the pharmacokinetic results (smaller C_{max} and AUC, higher Cl_p and similar Cl_D).

These preliminary results are important because they could be useful for further studies in various clinical situations where NAC may play a role. In addition to the possibility of acting as an anti-oxidant with potential advantages in the albumin synthesis [11], which is an important prognostic factor in uraemic patients, hydration associated to NAC is considered by some authors to be an efficacious manoeuvre for nephroprotection when iodinated contrast agents are used [17]. However results on the role of NAC in this context are at the moment uncertain, because various trials have failed to confirm this finding [18, 19]. For instance, a recent meta-analysis reported a reduction of the incidence of acutely increased serum creatinine after administration of intravenous contrast, but this finding was of borderline statistical significance and the authors were concerned with the heterogeneity among trials [20]. It is also not clear whether NAC could reduce the length of hospital stay in patients receiving intravenous contrast. For these reasons, a standard recommendation is premature, and new randomized trials on this topic are required. Nevertheless, a potential nephroprotective effect of NAC in patients with severe impairment of renal function undergoing radiographic examinations deserves a rigorous evaluation. It is true that uraemic patients, and especially dialysis patients, have only a small residual glomerular filtration, but even a protection of the remaining function is suitable to maintain water balance, a crucial point in the life quality of dialysis patients. It is also plausible that NAC could play a role in the correction of renal anaemia, particularly in erythropoietin-resistant anaemia. A study protocol concerning a potential effect of NAC in anaemic dialysis patients was prepared, and the study was ready to start but it has been secondarily cancelled for reasons that are unclear [21].

In conclusion, our results demonstrated that in dialysis patients 2 g of NAC given i.v. over 3 h is an acceptable dosage from the pharmacokinetic point of view, which in the short term did not produce side effects, at least in our small patient population. A steady state was reached on average after four dialysis sessions. A suitable administration modality is the heparin pump mixing NAC with the required heparin.

Acknowledgements We are grateful to Zambon Group S.p.A., Bresso, Milan, Italy, for assistance in the preparation of the protocol, supply of the necessary vials of *N*-acetylcysteine and financial support. We also thank Mrs Monia Fibbioli Crivelli for helping with data analysis.

References

1. Lindner A, Charra B, Sherrand DJ, Scribner BH (1974) Accelerated atherosclerosis in prolonged maintenance hemodialysis. N Engl J Med 290:697–701
2. Hakim R (1993) Clinical implications of hemodialysis membrane biocompatibility. Kidney Int 44:484–494
3. Alvarez V, Pulido R, Companero MR et al (1991) Differentially regulated cell surface expression of leukocyte adhesion receptors on neutrophils. Kidney Int 40:899–905
4. Amaout M, Hakim R, Todd R et al (1985) Increased expression of an adhesion-promoting surface glycoprotein in the granulocytopenia of hemodialysis. N Engl J Med 312:457–462
5. Kuwahara T, Markert M, Wauters JP (1988) Neutrophil oxygen radical production by dialysis membranes. Nephrol Dial Transplant 3:661–665
6. Nguyen AT, Lethias C, Zingraff J et al (1985) Hemodialysis membrane-induced activation of phagocyte oxidative metabolism detected in vivo and in vitro within micro amounts of whole blood. Kidney Int 28:158–167
7. Kolb G, Noiting C, Eckle I et al (1985) The role of membrane contact in hemodialysis-induced granulocyte activation. Nephron 57:64–68
8. Hirnmelfarb J, Ault KA, Holbrook D et al (1993) Intradialytic granulocyte reactive oxygen species production: a prospective, crossover trial. J Am Soc Nephrol 4:178–186
9. Becker BN, Himmelfarb J, Henrich WL et al (1997) Reassessing the cardiac risk profile in chronic hemodialysis patients: a hypothesis on the role of oxidant stress and other non-traditional cardiac risk factors. J Am Soc Nephrol 8:475–486
10. Boaz M, Matas Z, Biro A et al (1999) Serum malondialdehyde and prevalent cardiovascular disease in hemodialysis. Kidney Int 56:1078–1083
11. Suliman ME et al (2000) Hyperhomocysteinemia. Nutritional status and cardiovascular disease in hemodialysis patients. Kidney Int 57:1727–1735
12. Martindale (1999) The complete drug reference, 32nd edn. K. Parfitted, pp 1052–1054

13. Santangelo F, Witko-Sarsat V, Drüeke T, Descamps-Latscha B (2004) Restoring glutathione as a therapeutic strategy in chronic kidney disease. Nephrol Dial Transplant 19:1951–1955
14. Tepel M, Van der Giet M, Statz M, Jankowski J, Zidek W (2003) The antioxidant acetylcysteine reduces cardiovascular events in patients with end-stage renal failure. Circulation 107:992–995
15. Holdiness MR (1991) Clinical pharmacokinetics of N-acetylcysteine. Clin Pharmacokinet 20:123–134
16. Jones AL, Jarvie DR, Simpson D, Hayes PC, Prescott LF (1997) Pharmacokinetics of N-acetylcysteine are altered in patients with chronic liver disease. Aliment Pharmacol Ther 11(4):787–791
17. Tepel M, Van der Giet M, Schwarzfeld C, Laufer U, Liermann D (2000) Prevention of radiographic-contrast-agent-induced reductions in renal function by acetylcysteine. N Engl J Med 343:180–184
18. Durham JD, Caputo C, Dokko J et al (2002) A randomized controlled trial of N-acetylcysteine to prevent contrast nephropathy in cardiac angiography. Kidney Int 62:2202–2207
19. Allaqaband S, Tumuluri R, Malik AM et al (2002) Prospective randomized study of N-acetylcysteine, fenoldopam, and saline for prevention of radiocontrast-induced nephropathy. Catheter Cardiovasc Interv 57:279–283
20. Pannu N, Manns B, Lee H, Tonelli M (2004) Systematic review of the impact of N-acetylcysteine on contrast nephropathy. Kidney Int 65:1366–1374
21. Drüeke TB, Descamps-Latscha B, Locatelli F (2003) Stopping a medical research project for financial reasons. Nephrol Dial Transplant 18:1982–1983

invito 2000

ENCL. 2

Eur Respir J 2000; 16: 933–939
Printed in UK – all rights reserved

Regulation of lipopolysaccharide-mediated interleukin-1β release by *N*-acetylcysteine in THP-1 cells

M. Parmentier, N. Hirani, I. Rahman, K. Donaldson, W. MacNee, F. Antonicelli

Regulation of lipopolysaccharide-mediated interleukin-1β release by N-acetylcysteine in THP-1 cells. M. Parmentier, N. Hirani, I. Rahman, K. Donaldson, W. MacNee, F. Antonicelli. ©ERS Journals Ltd 2000.

ABSTRACT: Increased levels of inflammatory cytokines such as interleukin (IL)-1 and IL-8 occur in the bronchoalveolar lavage fluid in various lung diseases. Cytokine gene expression is controlled by transcription factors such as nuclear factor-κB (NF-κB) which can be activated by a number of stimuli including the oxidants prevent. It was hypothesized that lipopolysaccharide (LPS)-induced IL-1β secretion may be modulated by the intracellular thiol redox status of the cells.

The effect of the antioxidant compound, *N*-acetyl-L-cysteine (NAC), on IL-1β release and regulation of NF-κB in a human myelo-monocytic cell line (THP-1) differentiated into macrophages was studied.

LPS (10 μg·mL^{-1}) increased IL-1β release at 24 h compared to control levels ($p<0.001$). NAC (5 mM) also enhanced LPS-induced IL-1β release from THP-1 cells ($p<0.001$). In addition, treatment of cells with cycloheximide, an inhibitor of protein synthesis, inhibited the NAC-mediated IL-1β release. Under the same conditions, NF-κB binding was activated by LPS and NAC increased this LPS-mediated effect. Western blot analysis revealed that NAC treatment leads to an increase in p50 and p65 protein synthesis.

These data indicate that *N*-acetyl-L-cysteine modulates interleukin-1β release by increasing levels of the homo- and heterodimeric forms of nuclear factor-κB.

Eur Respir J 2000; 16: 933–939.

Respiratory Medicine Unit, Dept of Medicine, University of Edinburgh Medical School, Edinburgh, UK.

Correspondence: W. MacNee, Respiratory Medicine Unit, Edinburgh Lung in the Environment Group Initiative, Colt Research Laboratories, Wilkie Building, Medical School, Teviot Place, Edinburgh EH8 9AG, UK. Fax: 44 1316511558

Keywords: *N*-Acetyl-L-Cysteine, interleukin-1β, lipopolysaccharide, nuclear factor-κB, THP-1 cells

Received: February 24 2000
Accepted after revision August 8 2000

This work was supported by SMB Pharmaceuticals, Brussels, Belgium; the EUROLUNG Project, EU, BIOMED 2 Grant No. BMH4-CT96-0152; the Foundation Bettencourt Schueller, Paris and the Colt Foundation, New Lane, Havant, Hampshire, UK.

Multiple inflammatory events are involved in the development of lung injury. Part of this inflammatory response is the release of an array of cytokines [1–3]. The regulation of these inflammatory cytokines in response to diverse stimuli is controlled at the level of gene transcription. A crucial transcription factor that regulates the expression of several cytokines is nuclear factor-κB (NF-κB). A NF-κB consensus site is present in the promoter region of the interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor (TNF) genes [4]. Since a large range of inflammatory genes are induced by activated NF-κB, it has been proposed that activation of this transcription factor plays a central role in inflammatory processes, and activation of NF-κB has been implicated in a wide range of inflammatory diseases such as asthma and acquired immune deficiency syndrome [5–7].

NF-κB is a member of the Rel family of proteins, a novel family of ubiquitous transcription factors sharing a common structural motif for deoxyribonucleic acid (DNA) binding [4]. NF-κB was first identified as a nuclear factor that binds the decameric DNA sequence 5′-GGGAC-TTTCC-3′. NF-κB is a cytosolic homo-heterodimer consisting of p65 and p50 subunit proteins, each having specific affinity for different decameric binding sites fitting the IκB motif [8]. Inactive NF-κB is localized in the cytoplasm due to binding of the inhibitory protein (IκB). A primary event in the activation of NF-κB is phosphorylation, ubiquitination and degradation of IκB by specific IκB kinases [9–11]. This allows translocation of activated NF-κB from the cytoplasm to the nucleus.

The levels of many pro-inflammatory cytokines are increased in lung diseases. However, the mechanism of this upregulation is currently unknown. It has been shown that cytokines such as IL-8 are transcriptionally regulated by the redox state of the cell [12]. Constitutive expression of IL-1β is kept under tight control in healthy tissues and this is an important intrapulmonary cytokine in the mediation of lipopolysaccharide (LPS)-induced effects in the lung [13, 14]. Despite numerous reports regarding the importance of IL-1β in the cytokine network, very little is known about the molecular mechanisms governing its regulation. In the present study, the molecular regulation of IL-1β was investigated. Whether this regulation is under redox control and, in particular, the effect of intracellular thiol status was also studied using the compound *N*-acetyl-L-cysteine (NAC), an antioxidant drug. Hence, this study was designed to characterize the molecular mechanism of LPS-mediated regulation of IL-1β by NAC in a human myelo-monocyte-derived macrophage cell line (THP-1).

Materials and methods

Unless otherwise stated, all of the biochemical reagents used in the present study were purchased from Sigma Chemical Co. (Poole, UK) and cell culture media from Gibco (Paisley, UK).

Cell culture

THP-1 cells were maintained in suspension in Roswell Park Memorial institute (RPMI)-1640 medium containing 10% foetal calf serum. For experiments, the cells were plated in six-well culture dishes at a density of 1×10^6 cells·mL^{-1}. Differentiation of THP-1 monocytes into macrophages was by overnight incubation with phorbol myristate acetate (PMA) at a concentration of 10 μM. Differentiated cells adhered to the flask, whereas undifferentiated monocytic cells remained in suspension and were removed by washing with prosphate-buffered saline (PBS, pH 7.4). Adherent macrophage-like cells were incubated in serum-free RPMI-1640 medium.

Cell treatment

Cells were incubated in serum-free medium alone (control) or with LPS (10 μg·mL^{-1}). The effects of NAC (1, 5 and 10 mM) and glutathione monoethyl ester (GSHMEE; 5 mM) on IL-1β release from THP-1 cells were studied without or with coincubation with LPS (10 μg·mL^{-1}) for 24 h. Cycloheximide (CHX) (1 μg·mL^{-1}) and okadaic acid (OA) (0.1 μM) were introduced at 60 and 90 min, respectively, before addition of LPS (10 μg·mL^{-1}) and the antioxidant NAC (5 mM).

Preparation of nuclear extracts

After LPS and NAC treatment for 24 h, the medium overlying the cells was harvested for measurement of cytokine secretion and replaced with ice-cold PBS. THP-1 cells were harvested by scraping, followed by centrifugation at 1,000 × g. Nuclear extracts were prepared using the method of STAAL *et al.* [7].

Electrophoretic mobility shift assay and supershift assay

Binding reactions were established in 20 μL using 1.5 or 3 μg (4 and 24 h respectively) nuclear extract protein and 0.25 mg·mL^{-1} polydeoxyinosine-deoxycytidine in binding buffer (Promega) per reaction. In the binding reaction, the nuclear extracts were incubated with a γ-^{32}P-adenosine triphosphate end-labelled double-stranded NF-κB consensus oligonucleotide (Promega, Southampton, UK), produced using T4 polynucleotide kinase, for 20 min at room temperature. For the supershift assays, the nuclear extracts were first incubated with the appropriate antibody (rabbit antihuman NF-κB p50 and p65, AHP 287 and AHP 288 (Serotec, Oxford, UK); 2 μL) in a concentration of 1 μg·mL^{-1} for 3 h at 4°C. Preimmune rabbit serum was used as a control antibody. Samples were loaded and electrophoresed through a 6% polyacrylamide gel at a constant voltage of 180. Gels were then dried and autoradiography was performed.

Western blot analysis

THP-1 cells were lysed in buffer containing 10 mM *N*-2-hydroxyethylpiperazine–*N*-2-ethanesulphonic acid (HEPES; pH 7.8) 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM ethylenediamine tetra-acetic acid (EDTA), 0.4 mM phenylmethyl sulphonyl fluoride, 0.2 mM NaF, 1 mM sodium orthovanadate, 0.3 mg·mL^{-1} leupeptin and 10% Nonidet P-40. Protein (70 μg) was loaded, using Laemmli buffer, on to a 10% polyacrylamide gel and then transferred to nitrocellulose. After saturation in Blotto (5% dry milk powder and 0.05% Tween 20 in tris(hydroxymethyl)aminomethane (Tris)-buffered saline) for 1 h, the blot was probed with different rabbit antibodies dependent on the protein being analysed (anti-p50 and -p65 and anti-IκB-α (sc-203; Santa Cruz)). A secondary goat-anti-rabbit antibody (Scottish Antibody Production Unit, Edinburgh, UK) conjugated to horseradish peroxidase was added and gels were developed using chemiluminescence and autoradiography.

Isolation of ribonucleic acid and reverse transcription

Ribonucleic acid (RNA) was isolated from THP-1 cells using TRIZOL reagent (Life Technologies, Paisley, UK). Total RNA was reverse transcribed according to the manufacturer's instructions (8025SA; Life Technologies). The resultant complementary DNA (cDNA) was stored at -20°C until required.

Analysis of interleukin-1β messenger ribonucleic acid by polymerase chain reaction

Oligonucleotide primers were chosen using the published sequence of human IL-1β cDNA [15] and β-actin [16]. The primers for IL-1β and β-actin were synthesized by MWG Biotech (Milton Keynes, UK). The sequences of the primers used in the polymerase chain reaction (PCR) were as follows: IL-1β sense: 5′-ATGGCAGAAGTACCTGAGCTCGC-3′; IL-2β antisense: 5′-TAACTGACTTCACCATGCAATTTGTG-3′; β-actin sense: 5′-CCACCAACTGGGACGACATG-3′; and β-actin antisense: 5′-GTCTCAAACATGATCTGGGTCATC-3′. The reverse transcribed messenger RNA (mRNA) mixture (3 μL) was added directly to the PCR mixture and used for the PCR reactions. The IL-1β PCR conditions were 10 min at 94°C followed by 35 cycles of: 45 s at 94°C, 45 s at 52°C, 120 s at 72°C, and a final extension with 1 unit of *Taq* DNA polymerase (Promega) for 10 min at 72°C. The β-actin PCR conditions were 10 min at 94°C followed by 35 cycles of: 60 s at 94°C, 60 s at 60°C, and 60 s at 72°C and a final extension with 1 unit of *Taq* DNA polymerase for 5 min at 72°C. The identity of the resulting PCR-amplified DNA fragment was confirmed by DNA sequencing. Bands were visualized and scanned using a white ultraviolet transilluminator photometer (Orme Technologies, Cambridge, UK). Levels of IL-1β mRNA (801 base pairs (bp)) were expressed as a percentage relative to the intensity of the β-actin bands (121 bp).

Enzyme-linked immunosorbent assay for interleukin-1β

The enzyme-linked immunosorbent assay (ELISA) was performed as previously described [17]. All plates were read on a microplate reader (Dynatech MR 5000, Aldermaston, Berkshire, UK) and underwent computer-assisted analysis (Assay ZAP, Blosoft, Cambridge, UK). Typically, standard curves generated with this ELISA were linear in the range 50–2,500 pg·mL^{-1} IL-1β. Only assays yielding standard curves with a calculated regression coefficient of >0.95 were used for further analysis.

Statistical analysis

Data are expressed as mean±SEM. Data comparison was carried out using analysis of variance followed by the Tukey *post hoc* test for multigroup comparisons. The software package Instat 2 (GraphPad; San Diego, CA, USA) was used for this analysis. A p-value of <0.05 was regarded as significant.

Results

Effects of lipopolysaccharide, N-acetyl-L-cysteine and glutathione monoethyl ester on interleukin-1β release from THP-1 cells

At a concentration of 10 μg·mL^{-1}, LPS caused a two-fold increase in IL-1β release after incubation for 24 h (fig. 1) but no change after 4 h (data not shown). In order to analyse the effect of antioxidant thiols on IL-1β release, THP-1 cells were incubated with NAC for 24 h. NAC alone had no effect on IL-1β release from THP-1 cells (data not shown). Coincubation of NAC, at a concentration of 5 mM, with LPS increased secretion of IL-1β into the culture medium by 300% (fig. 1). Under these conditions, IL-1β was still undetectable after incubation for 4 h, suggesting that the IL-1β release occurred at a later time point. The effect of NAC on LPS-induced IL-1β secretion was bimodal and dose-dependent. At a concentration of 1 mM, NAC did not enhance IL-1β secretion, whereas higher concentrations (5 and 10 mM) produced the described effect. The increase in LPS-mediated IL-1β release observed with NAC was not reproduced using the thiol compound GSHMEE (fig. 1).

Effect of cycloheximide and okadaic acid on N-acetyl-L-cysteine-induced interleukin-1β release from THP-1 cells

In order to investigate the mechanisms by which NAC enhances LPS-mediated IL-1β release, cells were pretreated with CHX (1 μg·mL^{-1}) to prevent protein synthesis.

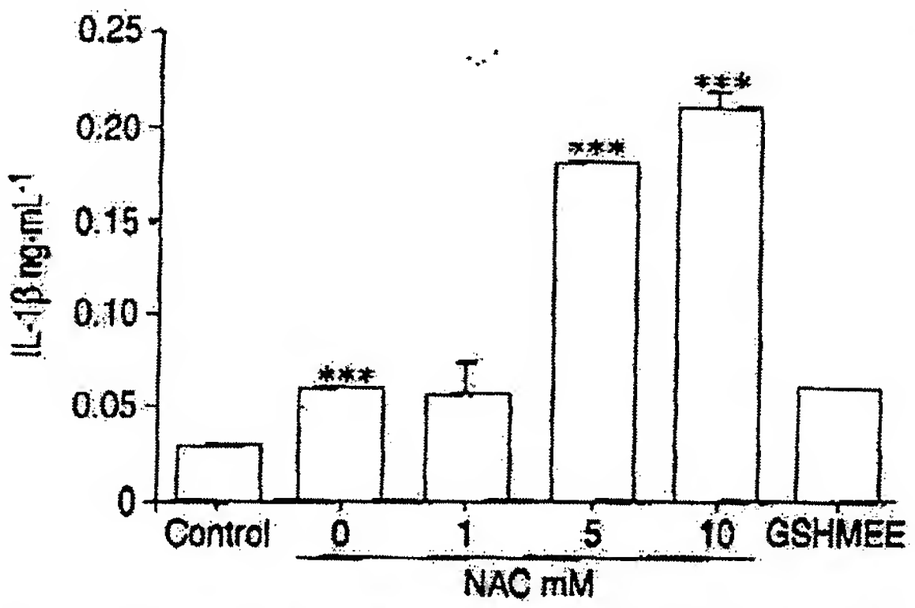

Fig. 1. – Effects of lipopolysaccharide (LPS), glutathione monoethyl ester (GSHMEE) and *N*-acetyl-L-cysteine (NAC) on interleukin-1b (IL-1β) release by THP-1 cells. Cells were incubated in serum-free medium alone (control) or with LPS (10 μg·mL^{-1}). LPS-stimulated cells were coincubated with or without NAC (1, 5 or 10 mM) or GSHMEE (5 mM) for 24 h. IL-1β accumulation in the medium was measured by enzyme-linked immunosorbent assay. Data are presented as mean±SEM (n=5). ***: p<0.001 *versus* control.

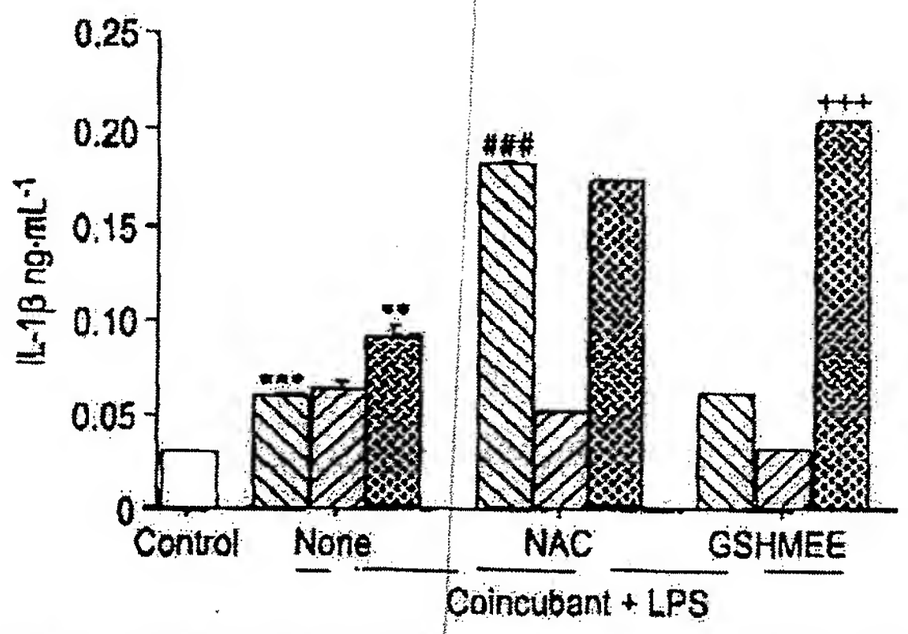

Fig. 2. – Effects of cycloheximide (CHX) or okadaic acid (OA) on *N*-acetyl-L-cysteine (NAC)-induced interleukin-1β (IL-1β) release by THP-1 cells. Cells were pretreated without (▨) or with CHX (1 μg·mL^{-1}) for 60 min (▩) or OA (0.1 μM) for 90 min (▩). The cells were then incubated in serum-free medium alone (control; no pretreatment) or with lipopolysaccharide (LPS; 10 μg·mL^{-1}). LPS-stimulated cells were coincubated with or without NAC or GSHMEE (both 5 mM) for 24 h. IL-1β accumulation in the medium was measured by enzyme-linked immunosorbent assay. Data are presented as mean±SEM (n=3). ***: p<0.001 *versus* control; **: p<0.01 *versus* LPS only; ###: p<0.001 *versus* LPS only; +++: p<0.001 *versus* LPS plus GSHMEE.

Figure 2 shows that a 60-min CHX pretreatment had no effect on LPS-mediated IL-1β release. However, the effect of NAC on LPS-induced IL-1β release was totally abolished by inhibiting protein synthesis. One effect of NAC is to increase intracellular glutathione levels [18, 19]. Thus it was decided to investigate whether the inhibitory effect of CHX was due specifically to inhibition of glutathione. Cells pretreated with CHX were incubated in the presence of the thiol-replenishing compound GSHMEE at a concentration of 5 mM. Figure 2 shows that, under these conditions, no increase in IL-1β level was observed, demonstrating that NAC-induced increased IL-1β release cannot be restored by addition of GSHMEE alone. This result is consistent with the observation that GSHMEE had no effect on LPS-mediated IL-1β release (fig. 1). Next, it was examined whether the NAC-mediated activation of IL-1β involved a phosphorylation step. Preincubation with OA, a specific inhibitor of serine/threonine phosphatases 1 and 2A, for 90 min did not modify the effect of NAC on LPS-induced IL-1β release from THP-1 cells (fig. 2). This result suggests that the effect of NAC on IL-1β release is not through kinase activation.

Effects of lipopolysaccharide, N-acetyl-L-cysteine on interleukin-1β messenger ribonucleic acid expression

Having demonstrated that NAC enhances LPS-induced IL-1β release, it was sought to investigate this effect at the mRNA level by semiquantitative PCR. IL-1β mRNA expression after 4 and 24 h was measured using β-actin mRNA as a control (fig. 3). IL-1β mRNA levels were increased by incubation with NAC, predominantly after 4 h (250 and 125% increase compared to untreated and LPS-treated cells at 4 and 24 h respectively).

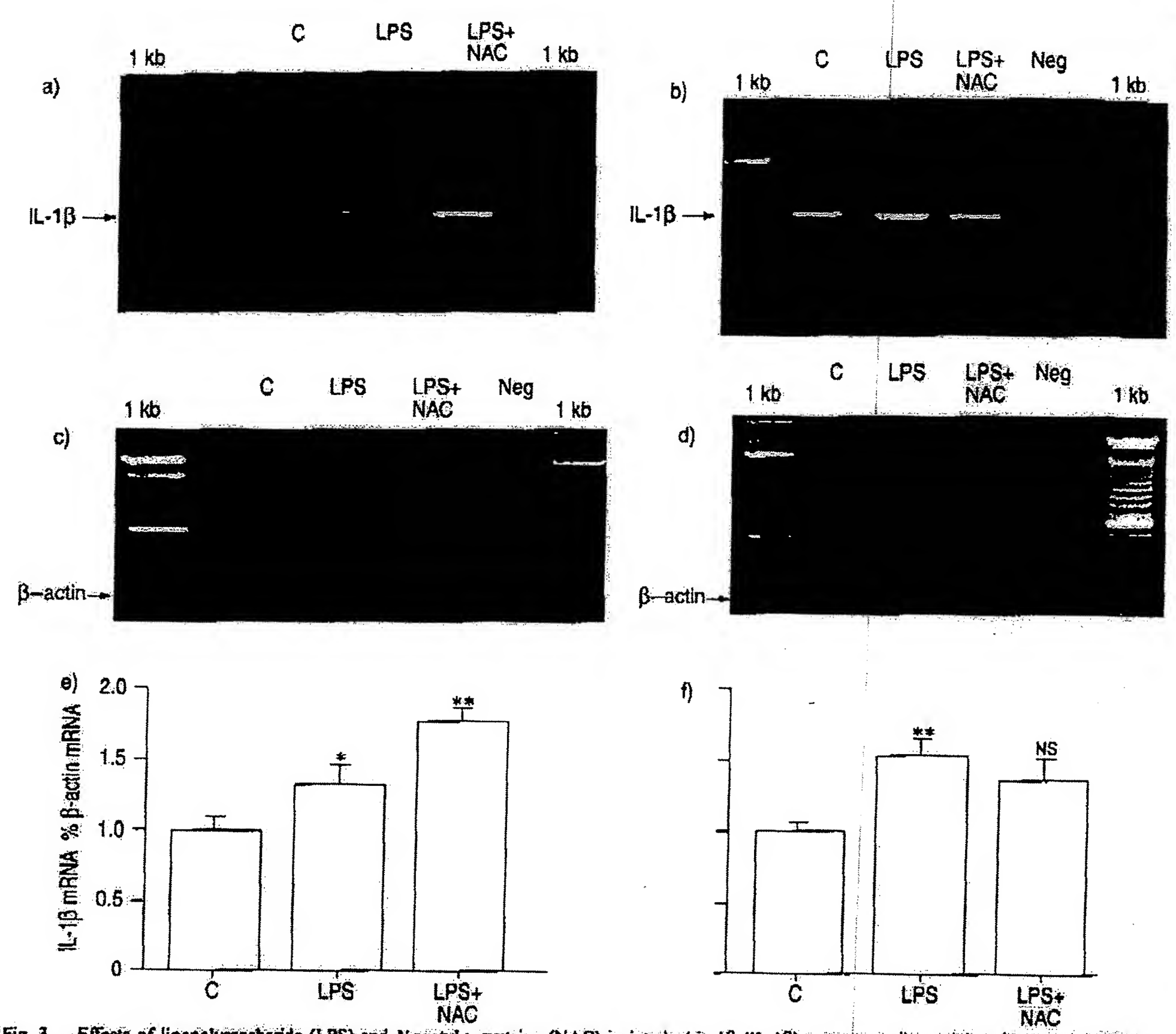

Fig. 3. – Effects of lipopolysaccharide (LPS) and *N*-acetyl-L-cysteine (NAC) in interleukin-1β (IL-1β) messenger ribonucleic acid (RNA) (mRNA) expression in THP-1 cells. Total RNA was isolated from cells incubated in serum-free medium alone (control (C)) or with LPS (10 μg·mL^{-1}) with or without NAC (5 mM) for: a, c, e) 4; and b, d, f) 24 h. RNA was reverse transcribed and used for polymerase chain reaction analysis of IL-1β mRNA as described in the *Isolation of ribonucleic acid and reverse transcription* and *Analysis of interleukin-1β messenger ribonucleic acid by polymerase chain reaction sections*. e, f) Numerical estimates of IL-1β mRNA levels (a, b) compared with β-actin bands (c, d) from the same sample. Data are expressed as mean±SEM (n=3). 1 kb: S (1 kilobase ladder); Neg: negative control, distilled water; NS: nonsignificant. *: $p<0.05$; **: $p<0.01$ *versus* control.

Role of nuclear factor-κB in the N-acetyl-L-cysteine-mediated regulation of interleukin-1β expression

In order to investigate the relationship between IL-1β release and NF-κB DNA-binding, the effect of NAC and LPS on the transcription factor NF-κB was determined. The presence of LPS in the culture medium increases NF-κB DNA-binding. This effect was observed after incubation for 4 and 24 h. The presence of NAC enhanced LPS-induced NF-κB DNA-binding (fig. 4). This result was verified using human blood-derived macrophages (data not shown). Similar to THP-1 cells, NAC (5 mM) treatment increased NF-κB DNA-binding by 166% compared to control.

To further explore the associated signalling events involved in the transcriptional regulation of IL-1β, whether NAC-mediated activation of NF-κB involved a phosphorylation step, phosphorylation of IκB being a key step in NF-κB activation, was examined. Using OA, an increase was observed in NF-κB DNA-binding (fig. 4), which followed degradation of IκB (fig. 5a-c), but produced no significant change in the NAC-mediated enhancement of LPS-stimulated activation, suggesting that a phosphorylation step is not the target of NAC.

The influence of CHX pretreatment on NAC-enhanced NF-κB nuclear binding was then assessed. The increase in NF-κB binding previously observed following treatment

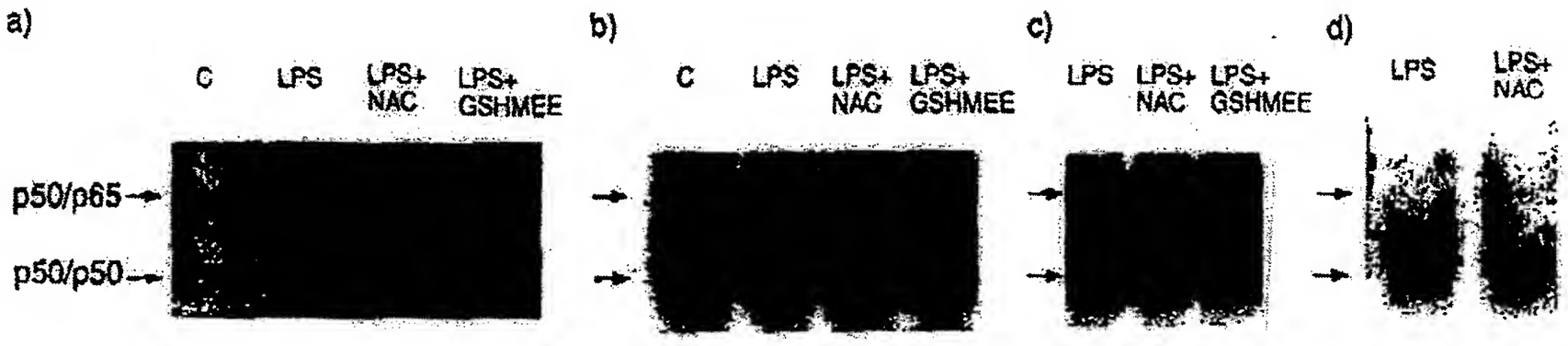

Fig. 4. – Nuclear factor-B binding in response to treatment with lipopolysaccharide (LPS) and *N*-acetyl-L-cysteine (NAC). Experiments were performed using 3 μg of protein from the nuclear extract of THP-1 cells. Cells were pretreated without (b) or with: c) cycloheximide (1 $\mu g \cdot mL^{-1}$) for 60 min; or d) okadaic acid (0.1 μM) for 90 min. The cells were then incubated in serum-free medium alone (control (C); no pretreatment) or with LPS (10 $\mu g \cdot mL^{-1}$) with or without NAC or glutathione monoethyl ester (GSHMEE) (both 5 mM) for: a) 4; and b, c, d) 24 h. The data are representative of three separate extractions.

with NAC was totally abolished when protein synthesis was inhibited (fig. 4).

Effect of N-acetyl-L-cysteine on p50 and p65 expression

It was then determined which combination of the p50/p65 proteins are involved in the NAC effect on LPS-induced NF-κB binding. A supershift assay (fig. 6) revealed that both the p50 and p65 proteins are involved, forming the homodimer p50/p50 and the heterodimer p50/p65. In order to obtain more information as to how NAC potentiates the LPS-induced NF-κB binding, the effect of NAC on the expression of the proteins p50 and p65 was analysed. Western blot analysis (fig. 5) indicated that both p50 and p65 levels were increased in cells treated with NAC, with no change in the expression of IκB (fig. 5).

Discussion

It has been reported previously that LPS activates the human monocytic cell line THP-1 using a serum-free system [20]. In the present report, it is shown that human THP-1 cells differentiated to macrophages with PMA respond to a low concentration of LPS (10 $\mu g \cdot mL^{-1}$) in the absence of serum, leading to the production of IL-1β. A further aim of the current study was to determine whether intracellular thiol status affected the molecular mechanisms of release of pro-inflammatory cytokine IL-1β. Serum-free conditions were chosen in order to avoid any antioxidant effect due to the serum. NAC alone does not modify IL-1β release. However, the present data show that THP-1 cells respond to NAC in dose-dependent fashion. At high concentration (5 and 10 mM), NAC enhances LPS-induced IL-1β release from THP-1 cells with no effect at lower concentration (1 mM). This effect occurred at the transcriptional level and is mediated in part by activation of NF-κB.

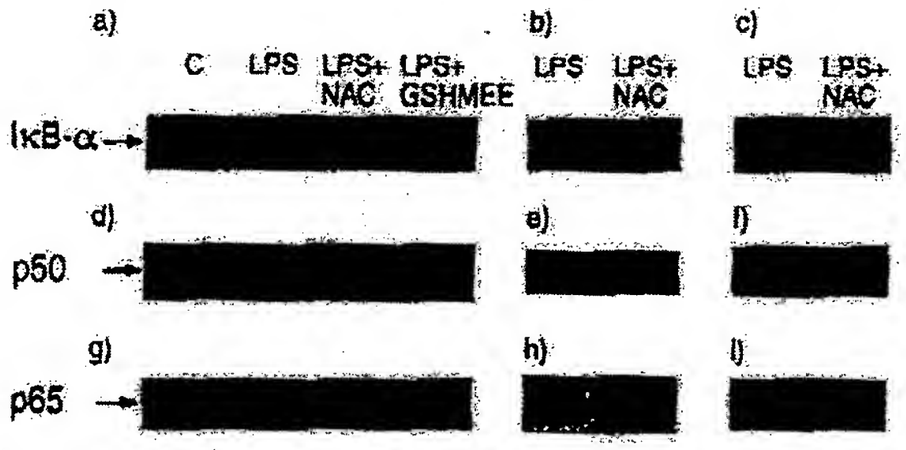

Fig. 5. – Determination of protein required for *N*-acetyl-L-cysteine (NAC)-mediated activation of nuclear factor-κB binding. Cells were pretreated without (a, d and g) or with: b, d, c, f, h) okadaic acid (0.1 μM) for 90 min; or c, f, i) cycloheximide (1 $\mu g \cdot mL^{-1}$) for 60 min. The cells were then incubated in serum-free medium alone (control (C); no pretreatment) or with lipopolysaccharide (LPS; 10 $\mu g \cdot mL^{-1}$). LPS-stimulated cells were coincubated with or without NAC or glutathione monoethyl ester (GSHMEE) for 24 h. Western blot analysis of matching extractions was performed for the determination of: a–c) the inhibitory protein (IκB-α); and d–f) p50; and p65 (g–i) protein content. Autoradiographs typical of three independent extractions are shown.

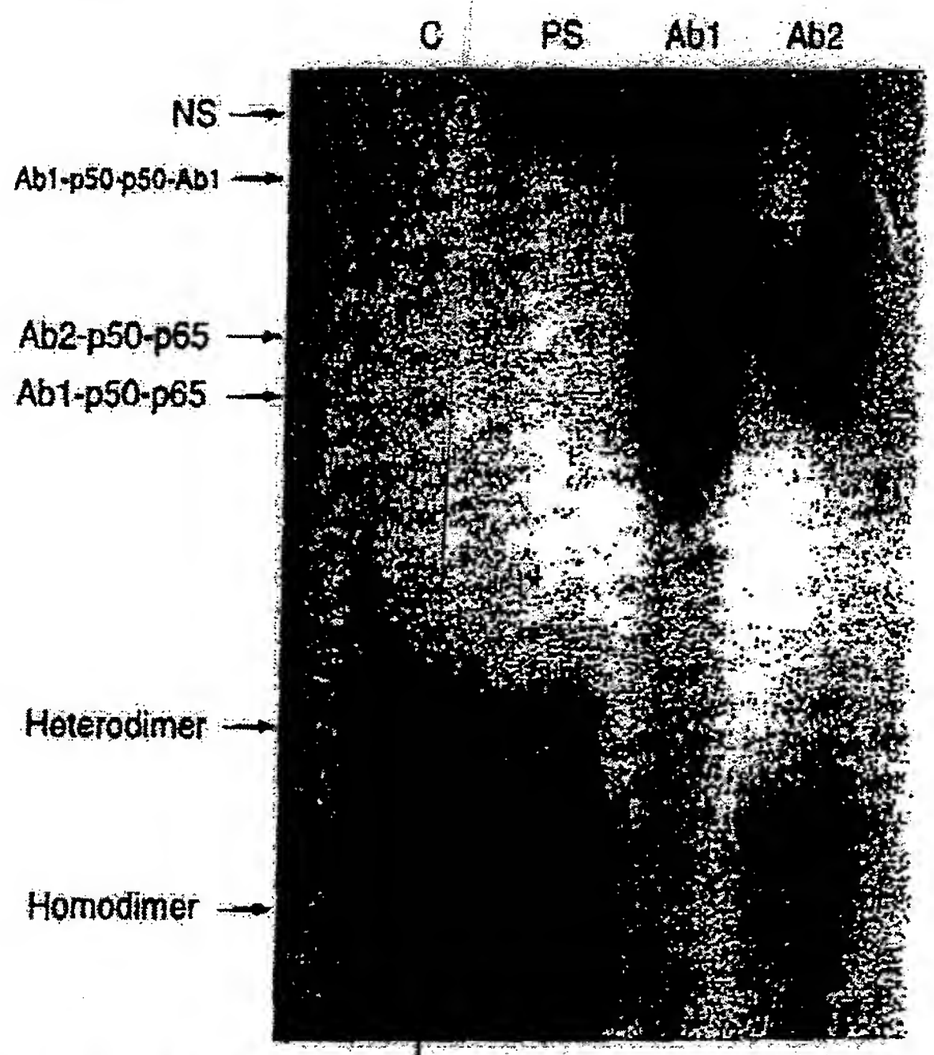

Fig. 6. – Identification of nuclear factor-κB (NF-κB) subunits by supershift assay. The addition of antibodies directed against potential components of the NF-κB complex resulted in supershift when antibodies to p50 or p65 were used. A nonspecific (NS) band was observed in the presence of preimmune serum (PS). Incubation with an antibody directed against p50 (Ab1) resulted in two supershifted bands corresponding to the homo- and heterodimer, whereas incubation with an antibody directed against p65 resulted in only one supershifted band corresponding to the heterodimer. Data are representatives of three separate extractions. C: control (LPS+NAC).

Previous studies have shown a correlation between the increase in NF-κB nuclear binding and production of IL-1β after LPS stimulation [21–24]. Other studies, in a variety of experimental systems, have shown that NAC effectively suppresses NF-κB activation induced by diverse stimuli [25, 26]. In Jurkat cells, for example, it has been shown that NAC at high concentration (20 mM) inhibits TNF-α-induced NF-κB activation, suggesting a role for reactive oxygen species (ROS) in the signalling mechanisms of NF-κB activation in these cells [27]. It appears, however, that the effect of NAC is dose-dependent such that low concentrations of NAC protect against LPS toxicity by decreasing hydrogen peroxide accumulation, whereas higher concentrations have the opposite effect [26]. In THP-1 cells, the dose-dependent effect of NAC on LPS-induced IL-1β showed a threshold between 1 and 5 mM. At a concentration of 5 mM, NAC enhances LPS-induced NF-κB binding. This is in accordance with the results of SUZUKI *et al.* [28] and BRENNAN *et al.* [27] who showed that NAC, at similar concentrations, did not inhibit the activation of NF-κB induced by stimulation with calyculin A in Jurkat cells or IL-1β in KB and Elu. NOB1 cells. Thus the effect of NAC on NF-κB activation is critically dependent upon concentration, cell type and stimulus. This is in agreement with the fact that the mode of action of NAC on signalling events is unclear. FOX and LEINGANG [29] showed that TNF-induced NF-κB activation was modified by NAC through an intermediate event.

In order to test the hypothesis that the effect of NAC might require an intermediate event, experiments were performed using OA, a potent inhibitor of serine/threonine phosphatases, and CHX, an inhibitor of protein synthesis. Phosphorylation is an important element in signal transduction for NF-κB [9, 10]. IκB dissociation involves its phosphorylation-controlled proteolytic degradation. ROS appear to play a role in regulating IκB phosphorylation through activation of the IκB kinase or inhibition of phosphatases. However, after 24-h incubation, OA did not modify the effect of NAC on LPS-induced IL-1β release from THP-1 cells and no changes were observed in IκB degradation. These results suggest that phosphorylation is not an intermediate event in NAC-induced NF-κB activation at 24 h and are consistent with the fact that IκB degradation is generally observed at an early time point. However, the same IL-1β level as that found in NAC-stimulated cells was reached in THP-1 cells cotreated with OA and GSHMEE, suggesting a possible inhibitory effect of NAC on the phosphatase.

By contrast, although it is clear that protein synthesis is not required for LPS-mediated release of IL-1β, the present study shows, for the first time, that CHX prevents NAC-enhanced IL-1β secretion, demonstrating the requirement for new protein synthesis in this phenomenon. MUHM *et al.* [30] showed modulation of the activation of the transcription factor NF-κB by intracellular reduced glutathione levels and by variations in extracellular cysteine supply. In THP-1 cells, it was found, in the present study, that 5mM NAC enhanced LPS-induced IL-1β release and NF-κB DNA-binding. However, the authors have also shown that NAC inhibits IL-8 release in THP-1 cells, confirming the potent antioxidant effect of this drug (unpublished data). Supplementation of cells with GSHMEE did not suppress the CHX inhibition of the NAC effect, suggesting that glutathione synthesis is not involved in the NAC activation of either IL-1β release or NF-κB binding. Furthermore, it was demonstrated that NAC increases both p50 and p65 expression and thus this is the probable mechanism of increased NF-κB activation. It could be argued that the NF-κB activation is not a direct effect of NAC but a secondary effect through IL-1β, since IL-1β itself is a strong inducer of NF-κB in monocytic cells [21]. However, the present finding of an increase in NF-κB binding after only a 4-h incubation with NAC, a time point at which IL-1β secretion was still undetectable, argues strongly in favour of a direct effect of NAC on NF-κB activation. Indeed, this effect was also observed after only 1 h with NAC (data not shown). This is in accordance with the observation that NAC has no effect on IL-1β-induced NF-κB activation in human vascular smooth muscle cells [31]. Furthermore, ZHANG *et al.* [32] showed a difference between signals transduced by IL-1β and LPS at the receptor level. Toll-like receptors (TLRs) 2 and 4 are present at the THP-1 cell membrane surface and are thought to transduce the LPS signal [32], whereas IL-1β binds to the IL^{-1} receptor. Thus NAC could interfere at a step between the TLR and MyD88 (an adaptor myeloid differentiation protein), which is a convergent point between the IL^{-1} and LPS signalling cascades. The resulting increase in IL-1β levels may then act in an autocrine loop mechanism, amplifying the effect.

Interleukin-1β is often considered to be an important pro-inflammatory cytokine, and, in this context, the present observation that *N*-acetyl-L-cysteine enhances interleukin-1β secretion may be interpreted as a potentially detrimental effect in patients with inflammatory diseases. However, this should be balanced against the known potent anti-oxidant, and hence anti-inflammatory, actions of *N*-acetyl-L-cysteine [27, 33, 34]. In other experiments, for example, it was demonstrated that, although the interleukin-1β level is enhanced, the interleukin-8 level is reduced by *N*-acetyl-L-cysteine (data not shown). Furthermore, under certain conditions, interleukin-1β itself may play an anti-inflammatory role. For example, in rats, an increased level of cytokine-induced chemoattractant (the interleukin-8 equivalent) was detected in lung lavage fluid, which was associated with a neutrophil-dependent acute oedematous lung leak. Under these conditions, pretreatment with interleukin-1 prevented lung leak in rats [35]. This balance between the potential pro-inflammatory and anti-inflammatory effects of *N*-acetyl-L-cysteine may be of direct relevance for patients with chronic inflammatory lung diseases.

References

1. Chollet-Martin S, Jourdain B, Gibert C, Elbim C, Chastre J, Gougerot-Pocidalo MA. Interactions between neutrophils and cytokines in blood and alveolar spaces during ARDS. *Am J Respir Crit Care Med* 1996; 153: 594–601.
2. Goodman RB, Strieter RM, Martin DP, *et al.* Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1996; 154: 602–611.
3. Baughman RP, Gunther KL, Rashkin MC, Keeton DA, Pattishall EN. Changes in the inflammatory response of the lung during acute respiratory distress syndrome: prognostic indicators. *Am J Respir Crit Care Med* 1996; 154: 76–81.

4. Rahman I, MacNee W. Role of transcription factors in inflammatory lung diseases. *Thorax* 1998; 53: 601–612.
5. Barnes PJ. Anti-inflammatory mechanisms of glucocorticoids. *Biochem Soc Trans* 1995; 23: 940–945.
6. Fiedler MA, Wernke-Dollries K, Stark JM. Mechanism of RSV-induced IL-8 gene expression in A549 cells before viral replication. *Am J Physiol* 1996; 271: L963–L971.
7. Staal FJT, Roederer M, Herzenberg LA. Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus. *Proc Natl Acad Sci USA*, 1990; 87: 9943–9947.
8. Baldwin AS Jr. The NF-κ B and I κ B proteins: new discoveries and insights. *Annu Rev Immunol* 1996; 14: 649–683.
9. Chen ZJ, Parent L, Maniatis T. Site-specific phosphorylation of IκBα by a novel ubiquitination dependent protein kinase activity. *Cell* 1996; 84: 853–862.
10. Didonato J, Mercurio F, Rosette C, *et al.* Mapping of the inducible IκB phophorylation sites that signal its ubiquitination and degradation. *Mol Cell Biol* 1996; 16: 1295–1304.
11. Henkel T, Machleidt T, Alkalay I, Kronke M, Benneriah Y, Bacuerle PA. Rapid proteolysis of I-kappa B-alpha is necessary for activation of transcription factor NF-κB. *Nature* 1993; 365: 182–185.
12. DeForge LE, Preston A, Takeuchi E, Kenney J, Boxer LA, Remick DG. Regulation of interleukin 8 gene expression by oxidant stress. *J Biol Chem* 1993; 268: 25568–25576.
13. Rose CE Jr, Juliano CA, Tracey DE, Yoshimura T, Fu SM. Role of interleukin-1 in endotoxin-induced lung injury in the rat. *Am J Respir Cell Mol Biol* 1994; 10: 214–221.
14. Xing Z, Jordana M, Kirpalani H, Driscoll KE, Schall TJ, Gauldie J. Cytokine expression by neutrophils and macrophages *in vivo*: endotoxin induces tumor necrosis factor-alpha, macrophage inflammatory protein-2, interleukin-1 beta, and interleukin-6 but not RANTES or transforming growth factor-beta 1 mRNA expression in acute lung inflammation. *Am J Respir Cell Mol Biol* 1994; 10: 148–153.
15. March CJ, Mosley B, Larsen A, *et al.* Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* 1985; 315: 641–647.
16. Ponte P, Ng SY, Gunning P, Kedes L. Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. *Nucleic Acids Res* 1984; 12: 1687–1696.
17. Donnelly S, Strieter R, Kunkel S, *et al.* Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet* 1993; 341: 643–647.
18. Issels RD, Nagele A, Eckert KG, Wilmanns W. Promotion of cysteine uptake and its utilization of glutathione biosynthesis induced by cysteamine and N-acetylcysteine. *Biochem Pharmacol* 1988; 37: 881–888.
19. Phelps DT, Deneke SM, Daley DL, Fanburg BL. Elevation of glutathione levels in bovine pulmonary artery endothelial cells by N-acetylcysteine. *Am J Respir Cell Mol Biol* 1992; 7: 293–299.
20. Lynn WA, Liu Y, Golenbock DT. Neither CD14 nor serum is absolutely necessary for activation of mononuclear phagocytes by bacterial lipopolysaccharide. *Infect Immun* 1993; 61: 4452–4461.
21. De Wit H, Dokter WH, Koopmans SB, *et al.* Regulation of p100 (NF-κB) expression in human monocytes in response to inflammatory mediators and Lymphokines. *Leukemia* 1998; 12: 363–370.
22. Haas M, Page S, Page M, *et al.* Effect of proteasome inhibitors on monocytic IκBα and β depletion, NF-kB activation and cytokine production. *J Leuk Biol* 1998; 63: 395–404.
23. Hatzigeorgiou DE, Geng J, Zhu B, *et al.* Lipophosphoglycan from Leishmania suppresses agonist-induced interleukin 1 beta gene expression in human monocytes *via* a unique promoter sequence. *Proc Natl Acad Sci USA* 1996; 93: 14708–14713.
24. Yoza BK, Hu JYQ, McCall CE. Protein-tyrosine kinase activation is required for lipopolysaccharide induction of interleukin 1β and Nf κ B activation, but not Nf κ B nuclear translocation. *J Biol Chem* 1996; 271: 18306–18309.
25. Pahan K, Sheikh FG, Namboodiri AM, Singh I. N-acetyl cysteine inhibits induction of NO production by endotoxin or cytokine stimulated rat peritoneal macrophages, C6 glial cells and astrocytes. *Free Radic Biol Med* 1998; 24: 39–48.
26. Sprong RC, Winkelhuyzen-Janssen AM, Aarsman CJ, van Oirschot JF, van der Bruggen T, Asbeck BS. Lowdose N-acetylcysteine protects rats against endotoxin-mediated oxidative stress, but high-dose increases mortality. *Am J Respir Crit Care Med* 1998; 157: 1283–1293.
27. Brennan P, O'Neill LAJ. Effects of oxidants and antioxidants on nuclear factor κB activation in three different cell lines: evidence against a universal hypothesis involving oxygen radicals. *Biochim Biophys Acta* 1995; 1260: 167–175.
28. Suzuki YJ, Mizuno M, Packer L. Signal transduction for nuclear factor-κ B activation. Proposed location of antioxidant-inhibitable step. *J Immunol* 1994; 153: 5008–5015.
29. Fox ES, Leingang KA. Inhibition of LPS-mediated activation in rat Kupffer cells by N-acetylcysteine occurs subsequent to NF-κB translocation and requires protein synthesis. *J Leukoc Biol* 1998; 63: 509–514.
30. Mihm S, Galter D, Droge W. Modulation of transcription factor NF-κB activity by intracellular glutathione levels and by variations of the extracellular cysteine supply. *FASEB* 1995; 9: 246–252.
31. Hoare GS, Marczin N, Chester AH, Yacoub MH. Role of oxidant stress in cytokine-induced activation of NF-κB in human aortic smooth muscle cells. *Am J Physiol* 1999; 277: H1975–H1984.
32. Zhang FX, Kirschning CJ, Mancinelli R, *et al.* Bacterial lipopolysaccharide activates nuclear factor-κB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. *J Biol Chem* 1999; 274: 7611–7614.
33. Matsumoto K, Hasimoto S, Gon Y, Nakayama T, Takizawa H, Horie T. N-acetylcysteine inhibits IL-1alpha induced IL-8 secretion by bronchial epithelial cells. *Respir Med* 1998; 92: 512–515.
34. Leff JA, Wilke CP, Hybertson BM, Shanley PF, Beehler CJ, Repine JE. Postinsult treatment with N-acetyl-l-cysteine decreases IL^{-1}-induced neutrophil influx and lung leak in rats. *Am J Physiol* 1993; 265: L501–L506.
35. Hybertson BM, Lee YM, Repine JE. Phagocytes and acute lung injury: dual roles for interleukin-1. *Ann NY Acad Sci* 1997; 832: 266–273.

Titre du document / Document title
Various forms of plasma cysteine and its metabolites in patients undergoing hemodialysis

Auteur(s) / Author(s)
WLODEK Przemyslaw J. [(1)] ; ICIEK Malgorzata B. [(2)] ; MILKOWSKI Andrzej [(1)] ; SMOLENSKI Olgierd B. [(1)] ;

Affiliation(s) du ou des auteurs / Author(s) Affiliation(s)
[(1)] Department of Nephrology, Rydygier Hospital, os. Zlotej Jesieni 1, 31-826 Cracow, POLOGNE
[(2)] Institute of Medical Biochemistry, Collegium Medicum, Jagiellonian University, M. Kopernika 7, 31-034 Cracow, POLOGNE

Résumé / Abstract
The thiol tripeptide glutathione (GSH) is particularly important as an antioxidative protector in the cells. GSH is also a form of storage and transport of cysteine. Under physiological conditions, the kidney plays an essential role in GSH biodegradation to free cysteine via γ-glutamyl cycle and subsequently in further metabolism of this sulfur amino acid. Our aim was to assess to what degree renal insufficiency affects the level of various cysteine forms and its metabolites (sulfates and sulfane sulfur compounds) in the plasma, and whole blood GSH levels. The concentrations of all the above mentioned sulfur compounds were measured in plasma of patients with end stage renal failure (ESRF) before and after dialysis and in a group of healthy controls. **In plasma of patients with ESRF before dialysis tendency towards significant elevation of cystine, protein-bound cysteine and sulfates levels was evident.** Simultaneously, a decrease of plasma level of sulfane sulfur compounds, products of anaerobic sulfur metabolism, and whole blood GSH concentration was found. As a consequence, the ratio between the reduced cysteine and the total cysteine concentration was markedly decreased. The dialysis session restore this ratio to the value observed in plasma of control individuals These findings indicate disturbances in the thiol-disulfide equilibrium and show a higher oxidation status in plasma of patients with ESRF than in healthy controls.

Revue / Journal Title
Clinica chimica acta **ISSN** 0009-8981 **CODEN** CCATAR

Source / Source
2001, vol. 304, n°1-2, pp. 9-18 (42 ref.)

Langue / Language
Anglais

Editeur / Publisher
Elsevier, Shannon, IRLANDE (1956) (Revue)